

**An examination of walking track analysis footprints of right-side unoperated limbs in rats  
prior to and following peripheral nerve injury and PEG fusion repair**

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in Neuroscience

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An examination of walking track analysis footprints of right-side unoperated limbs in rats prior to and following peripheral nerve injury and PEG fusion repair

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## **Abstract**

Peripheral nerve injury can result in long-lasting functional deficits in humans due to mammals' limited axon regenerative capacity. PEG fusion is a defined technique that fuses injured axons, resulting in morphological and electrophysiological continuity. The technology has been shown to restore lost behaviors due to peripheral nerve injury. The process involves an invasive surgical procedure on the left hind limb of female Sprague-Dawley rats that results in the severance of the sciatic nerve. Assessing behavioral recovery is perhaps the most important metric by which to quantify PEG fusion's success. Recovery is most accurately and best assessed using the Sciatic Functional Index walking track analysis, or SFI. This procedure involves having a rat run up a path with its paws inked, and subsequent analysis compares the print length, toe spread, and intermediate toe spread of the "normal" and unoperated side (right) with the "experimental" and operated side (left). In this paper, we examine if surgical intervention and sciatic nerve transection on the left, experimental limb in rats results in significantly altered measurements on the right, unoperated side compared to pre-op, baseline measurements. Comparisons are made between baseline normal prints, normal prints three days post-op, and normal prints 42 days post-op for PEG-fused animals. Differences may exist due to altered gait after operation, physiological effects of surgical intervention, or random variation in the SFI testing paradigm. Regardless, attempting to highlight such discrepancies can contribute to developing better models for measuring recovery following peripheral nerve injury in mammals. Such models would more accurately be able to predict the clinical translatability of potential treatments for these nerve injuries. Analysis of SFI measurements pre- and post-op suggests that surgical intervention on the left experimental hind limb of rats does result in statistically

significant differences in measured print length, toe spread, and intermediate toe spread post-op. In addition, we found that the differences between baseline and post-op measurements seem to become mitigated over time.

## **Introduction**

Methods to repair peripheral nerve crush and cut injuries are still under extensive investigation due to the limited regenerative capacity of mammalian axons and the dramatic behavioral deficits that result from such injuries (Campbell 2008). PEG fusion is one method that involves the fusion of severed axonal ends using the membrane fusogen polyethylene glycol, or PEG, and a specific sequence of bioengineered solutions (Riley et al. 2015). While morphological assessments, which may include the ability of the fused nerve to conduct CAPs or microscopic evaluations, are important in judging the success of nerve regeneration techniques, the ability of such processes to improve behavioral recovery is the most important metric. Behavioral recovery is commonly and most accurately assessed by the Sciatic Functional Index walking track analysis. Recovery in PEG-fused cut-severed sciatic nerves in rats has been shown to be significant, with scores returning close to baseline levels after 6-12 weeks (Bittner et al. 2012).

The Sciatic Functional Index is a procedure first developed by de Medinaceli et al. in 1982 that aimed to quantify the effects of peripheral nerve damage in animals. The SFI quantifies functional deficits in mammals by analyzing the change in footprint after injury. Rats are handled and trained to walk up an inclined path with their paws inked. The print length, toe spread, and intermediate toe spread are all measured and inserted into a formula (shown below) to yield a SFI score.

$$SFI = \left( \frac{NPL - EPL}{EPL} + \frac{ETS - NTS}{NTS} + \frac{EIT - NIT}{NIT} \right) * 73$$

(Carlton and Goldberg 1986)

Figure 1 shows the typical elements found in a footprint. The print length (PL) consists of the length of the footprint, measured from the base of the print to the third toe. The toe spread (TS) consists of the horizontal distance between the first and fifth toe. The intermediate toe spread (ITS) consists of the horizontal distance between the second and fifth toe. An SFI score of -100 indicates complete impairment, while an SFI score of 0 is normal (de Medinaceli et al. 1982, Varejao et al. 2001).

After sciatic injury, the injured print typically displays a much longer paw length, and much smaller toe and intermediate toe spreads compared to the normal print. Animals with no nerve damage can walk predominantly on their toes, while those with nerve damage are unable to support their own weight on their toes and end up placing their whole foot down, from ankle to toe. Such behavior results in a longer print length (de Medinaceli et al. 1982). Dellon and Dellon demonstrated that heavier rats exhibited a significantly greater PL, TS, and ITS than their lighter counterparts (Dellon and Dellon 1991). Further, it was observed on video that rats who had suffered nerve injury demonstrated shorter gait-stance duration on the injured leg than the uninjured leg (Dijkstra et al. 2000). These results suggest that the “normal” prints after injury might demonstrate some significant differences compared to pre-operative measurements. In this study, we try to demonstrate significant differences in pre- and post-op measurements by analyzing a multitude of SFI measurements from rats that were PEG fused. Such an analysis can highlight potential changes to the SFI paradigm that might contribute to more accurate and precise behavioral testing in the future.

## **Materials and Methods**

### *Surgical Procedures*

All experimental procedures were approved by, and performed in accordance with the standards set forth by the Institutional Animal Care and Use Committee at UT Austin. Female Sprague-Dawley weighing between 225-300g were used in this experiment. They were anesthetized with inhaled isoflurane/oxygen (4% at 1.5L/min, Handlebar Anesthesia, Austin, TX), and the left hind limb was shaved and disinfected with 10% iodine/povidone. During surgery, the rats were kept under at 2%, 1.5L/min with isoflurane/oxygen. The sciatic nerve was exposed by a 2 cm incision in the thigh of the left hind limb, parallel to the muscle fibers. These fibers were bluntly dissected away to reveal the sciatic nerve. Connective tissue surrounding the exposed nerve was cleaned away, and the sciatic was completely transected by precisely cutting the nerve at the lesion site with microdissection scissors. The area was flushed with a calcium-free hypotonic saline to open cut ends, and the nerve ends were trimmed and treated with methylene blue in distilled water. Then, the two nerve segments were closely apposed and connected using a series of 10-0 microsutures. Animals were then PEG-fused.

For PEG-fused animals, a 50% w/w solution of hypotonic polyethylene glycol was administered to the lesion site for 1-2 minutes, after which the microsutured ends were washed with Ca<sup>2+</sup>-containing Lactated Ringer's solution.

### *Behavioral Testing and Study Design*

Rats were handled and trained by blind testers at least one week before surgery and the start of behavioral testing, and baseline results were obtained two days before surgeries. Both the left and right hind paws of the rat were marked with blue and red ink, respectively. The rat was

then placed on an inclined wooden board 100mm in width and 5ft in length, lined with receipt paper. The rat would then run from the base of the board to its home cage at the top of the incline, and the tapes containing footprints were gathered and assigned scores by blind graders. A successful tape must contain three consecutive footprints of both the experimental and the normal footprints. Two successful tapes were obtained for each rat at each testing day. Rats were tested for behavioral recovery three days post-op, and weekly thereafter up to 42 days post-op.

The Sciatic Functional Index, or SFI, walking track analysis, which uses footprints to measure how well sciatic axons innervate distal muscle groups, was used in this experiment to assess behavioral recovery (de Medinaceli et al. 1982; Varejao et al. 2001). Rats were previously trained to walk up a wooden board into a cage. Three consecutive footprints from each limb were used to obtain the relevant measurements in the SFI formula, as described above (de Medinaceli et al. 1982): paw length (PL), toe spread (TS), and intermediate toe spread (ITS) measurements were made in millimeters. On every testing day, every rat therefore receives six measurements of each SFI parameter.

Each rat underwent a sciatic nerve transection operation on the left hind limb, and the baseline measurements of the normal paw prints were compared to the normal measurements three days post-op and 42 days post-op. Specifically, the baseline normal paw length, toe spread, and intermediate toe spread were compared to the normal paw length post-op by subtracting the post-op value from the baseline value. The differences between each set of measurements were compiled, and paired *t*-tests were run to determine if the differences between baseline and post-op normal measurements were statistically different from 0. Further, a Student's *t*-test was run between differences at 3d-PO and 42d-PO to determine if any changes between baseline and

post-op measurements become mitigated over time. Finally, a hypothetical comparison was conducted to examine how discrepancies between baseline and post-op normal measurements could potentially translate to final SFI scores. Theoretical SFI scores were calculated first using mean experimental post-op values and mean normal post-op values at 3d and 42d PO. Then, similar SFI scores were calculated with using experimental post-op values at 3d and 42d PO and mean baseline normal measurements substituted for mean post-op normal values at 3d and 42d.

## **Results**

The mean baseline values for PL, TS, and ITS for the normal side (Table 1), respectively were 19.93, 17.90, and 9.17mm. The mean 3d-PO values were 27.87, 19.79, and 10.37mm, and the mean 42d-PO values were 24.30, 18.13, and 9.33mm.

The mean baseline for PL, TS, and ITS for the experimental side (Table 2), respectively were 19.88, 18.10, and 9.01mm. The mean 3d-PO values were 32.83, 6.53, and 4.23mm, and the mean 42d-PO values were 29.44, 14.83, and 8.87mm.

The mean difference between baseline and 3d-PO normal PL, TS, and ITS (Table 3) were -7.93mm, -1.80mm, and -1.20mm respectively, and all of these results were significantly different than 0 (paired t-test,  $p<0.01$ ,  $p<0.05$ ). The mean difference between baseline and 42d-PO normal PL, TS, and ITS were -4.36mm, -0.23mm, and -0.16mm, and only the PL difference was significant (paired t-test,  $p<0.01$ ). The 3d-PO differences and the 42d-PO differences also significantly differed across all SFI elements (Student's t-test,  $p<0.01$ ).

The SFI scores calculated using mean normal baseline measurements and those calculated using post-op normal measurements differed by about ten points each (Table 4).

## **Discussion**



The normal measurements for PL, TS, and ITS pre- and post-op do significantly differ. Interestingly, the print length demonstrated the greatest and most significant difference between baseline and post-op measurements (Table 3). Dellon and Dellon suggested that nerve injury causes the contralateral hind limb to compensate for the loss of function in the injured limb (Dellon and Dellon 1992). That is, in the early stages of injury, the rat might utilize most of the uninjured foot rather than only its toes; such behavior would result in a longer PL, as seen 3d PO. In the same vein, rats tend to disproportionately step longer with their uninjured foot, and the increased time spent on the tape might also contribute to the difference in measurement (Dijkstra et al. 2000). As time progresses, however, it seems like the rat becomes accustomed to injury, and behavioral recovery progresses via the action of PEG fusion, resulting in a smaller difference between baseline and post-op measurements at 42 days PO (Table 3). It was suggested that rats can become accustomed to injury early, resulting in a lower discrepancy between baseline and post-op measurements (Chamberlain et al. 2000)

The difference between baseline and post-op measurements with regard to TS and ITS is markedly smaller, but such a result is probably due to the nature of the element in the SFI equation. The range of TS and ITS values is generally much smaller than that of PL values, so it is not surprising that the discrepancy is lower. However, because the SFI equation still places a large emphasis on differences in TS and ITS, small differences in either one or both can have a large impact on the final SFI score for an animal on a given test day. Table 4 demonstrates the theoretical impact that a change in normal measurements can have on the final SFI score. If the normal mean baseline values were substituted for the post-op baseline values, the hypothetical SFI score would be about ten points lower. However, the resulting differences do not necessarily

reflect SFI differences in practice, as the SFI scores in each cell in Table 4 were calculated using averaged measured values from footprints from five different rats.

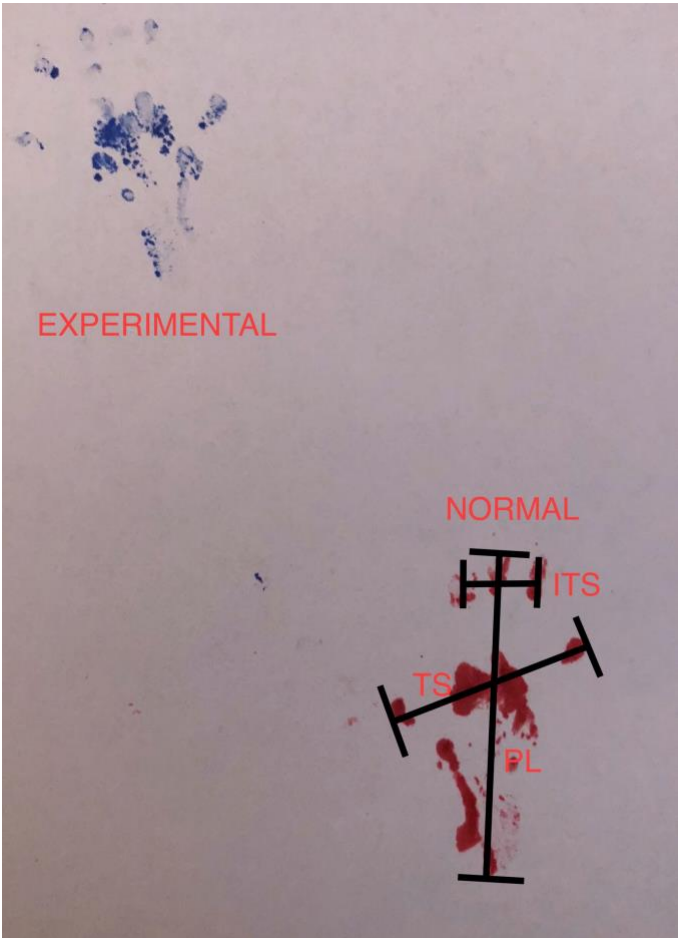
These results indicate that future considerations might be taken into account when calculating SFI scores for animals soon after peripheral nerve injury. Developing techniques to remedy such discrepancies can be difficult and perhaps may not even be needed because comparisons of behavioral recovery using SFI scores will generally use the post-operative results regardless, and any perceived discrepancies will be found among all treatment groups, thus mitigating the effects of pre- and post-op differences. However, enforcing tactics like observing more closely how the rat walks up the track (no slipping, no “compensation” on the uninjured limb) might result in more accurate measurements.

However, there are several limitations to this study. Perhaps most importantly, the vital factor of human error cannot not be overlooked when discussing the validity of the Sciatic Functional Index. SFI tapes are often graded by blind researchers, and one would be hard pressed to find two graders score one tape the exact same way, especially considering that SFI scores literally rely on millimeter differences in length. In addition, it is not at all uncommon to run several trials per rat to obtain valid prints, and these extra trials might also contribute to print variability. The tapes and footprints analyzed in this study may have suffered from a great deal of variation by the very nature of the SFI paradigm. Thus, it is difficult to make any definitive statements about the discrepancies observed, other than the fact that there were some significant differences between pre-op and post-op normal footprints. These differences could be due to the physical act of walking post injury, or they could be a result of variation within the experimental design. Future studies might solely investigate the phenomenon outlined in this study using a

larger and more controlled sample of rats. That is, a more accurate study would ensure that all the footprints were obtained using the same number of trials, and that all the rats were treated similarly for the sole purpose of discovering whether normal measurements change following intentional peripheral nerve injury.

**Figures**

Figure 1



**Tables**

Table 1

	Mean Baseline Value (mm)	3d-PO Mean Value (mm)	42d-PO Mean Value (mm)
Print Length (PL)	19.93	27.87	24.30
Toe Spread (TS)	17.90	19.79	18.13
Intermediate Toe Spread (ITS)	9.17	10.37	9.33

Table 2

	Mean Baseline Value (mm)	3d-PO Mean Value (mm)	42d-PO Mean Value (mm)
Print Length (PL)	19.88	32.83	29.44
Toe Spread (TS)	18.10	6.53	14.83
Intermediate Toe Spread (ITS)	9.01	4.23	8.87

Table 3

	3d-PO Mean Difference (mm)	42d-PO Mean Difference (mm)
Print Length (PL)	-7.93 !**	-4.36**
Toe Spread (TS)	-1.80 !*	-0.23
Intermediate Toe Spread (ITS)	-1.20 !*	-0.16

Table 4

	Using mean post-op normal measurements	Using mean baseline normal measurements
SFI-3d PO	-103.64	-114.37
SFI-42d PO	-29.63	-38.48

## Legends

Figure 1: Snapshot of a pair of baseline footprints on an SFI tape. The left, blue print is the experimental print and corresponds to the limb that will be operated on. The right, red print is the normal print and will remain uninjured in the PEG fusion paradigm. The elements of the SFI are marked on the normal print. PL: print length; TS: toe spread; ITS: intermediate toe spread.

Table 1: Mean measurements of SFI elements taken from a sample of 30 footprints for the NORMAL (right) limb of PEG-fused rats. The baseline values are those measured before surgery, 3d-PO values are those measured at the 3<sup>rd</sup> postoperative day, and 42d-PO values are those measured at the 42<sup>nd</sup> postoperative day.

Table 2: Mean differences between SFI elements taken from a sample of 30 footprints for the NORMAL (right) limb of PEG-fused rats. Differences were calculated by subtracting post-op measurements from baseline measurements. Thus, the negative values indicate that the baseline measurements were smaller than the post-op measurements. \*\* denotes that the difference in measurements is statistically different from zero (paired *t*-test,  $p < 0.01$ ). \* denotes that the difference in measurement is statistically different from 0 (paired *t*-test,  $p < 0.05$ ). ! indicates that the 3d-PO and 42d-PO measurements are statistically significant from one another (Student's *t*-test,  $p < 0.01$ ).

Table 3: Mean measurements of SFI elements taken from a sample of 30 footprints for the EXPERIMENTAL (left) limb of PEG-fused rats. The baseline values are those measured before surgery, 3d-PO values are those measured at the 3<sup>rd</sup> postoperative day, and 42d-PO values are those measured at the 42<sup>nd</sup> postoperative day.

Table 4: Hypothetical comparison between SFI scores calculated using both normal and experimental mean post-op measurements at 3d and 42d and SFI scores calculated using experimental mean post-op values at 3d and 42d and mean normal baseline measurements substituted for post-op normal measurements.

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